TLR4 is a novel determinant of the response to paclitaxel in breast cancer

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Abstract

Overexpression of Toll-like Receptor-4 (TLR4) in human tumors often correlates with chemoresistance and metastasis. We found that TLR4 is overexpressed in the majority of clinical breast cancer (BC) samples and in 68% of the examined BC lines. TLR4 is activated by lipopolysaccharide (LPS) and other ligands including the widely-used drug paclitaxel (PXL). LPS is frequently used to show a tumor-promoting role of TLR4 although this bacterial component is unlikely to be found in BC environment. We reasoned that PXL-dependent activation of TLR4 is more relevant to BC chemoresistance that could be mediated by activation of the NF-κB pathway leading to upregulation of pro-survival genes. To test this hypothesis, we correlated TLR4 expression with resistance to PXL in two modified BC lines with either depleted or overexpressed TLR4 protein. Depletion of TLR4 in naturally overexpressing MDA-MB-231 cells downregulated pro-survival genes concomitant with 2–3 fold reduced IC_{50} to PXL *in vitro* and a 6-fold decrease in recurrence rate *in vivo*. Conversely, TLR4 overexpression in a negative cell line HCC1806 significantly increased expression of inflammatory and pro-survival genes along with a 3-fold increase of IC_{50} to PXL *in vitro* and enhanced tumor resistance to PXL therapy *in vivo*. Importantly, both tumor models showed that many PXL-upregulated inflammatory cytokines were co-induced with their receptors suggesting that this therapy induces autocrine tumor-promoting loops. Collectively, these results demonstrate that paclitaxel not only kills tumor cells but also enhances their survival by activating TLR4 pathway. These findings suggest that blocking TLR4 could significantly improve response to PXL therapy.

Keywords

TLR4; chemoresistance; breast cancer; paclitaxel

Introduction

Chemoresistance is the major obstacle to successful treatment of metastatic cancers. The two well-recognized mechanisms of chemoresistance are overexpression of drug transporters that decrease intracellular concentration of the drug (1) and preexisting mutations that decrease drug binding to target proteins (2). In addition to these intrinsic mechanisms, therapeutic efficacy can be reduced by chemotherapy-induced upregulation of inflammatory

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and pro-survival genes(3–5). The goal of this study was to delineate the latter mechanism that enables tumor cells to escape cytotoxic therapy through acquisition of a more aggressive phenotype.

Chemotherapy induction of tumor-promoting pathways can be illustrated by response of malignant cells to taxanes. Paclitaxel (PXL),the active component of taxanes, is a common drug used against various solid tumors (6). PXL induces apoptosis by over-stabilizing microtubules (7), which leads to cell arrest at the G2/M phase (8). Although the therapeutic utility of PXL has been improved by formulating it as nanoparticles(nab-PXL) (9), the overall response of breast cancer (BC) patient seven to this advanced therapy remains below 35% (10). The main reason for low response rate is tumor recurrence after cessation of therapy, which might relate to PXL-mediated induction of inflammatory mediators including TNF-α (11), IL-1β(12), IL-8(13), IL-6 (14)and VEGF-A (3, 4). In addition to inflammatory mediators, PXL also upregulates pro-survival proteins including XIAP (15), Bcl-2 (16), Akt (17), and Bcl-xL (4, 15). Cumulatively, these studies suggest that tumor cells respond to PXL by activating two conflicting pathways: one induced by over-stabilizing microtubules which leads to apoptosis, and the other induced by a currently undefined receptor that promotes tumor cell survival through upregulation of inflammatory and pro-survival genes.

Based on the transcriptional profile induced by PXL, we postulated that the likely candidate for transmitting PXL signaling is Toll-like Receptor-4 (TLR4). This hypothesis is based on multiple lines of evidence. First, PXL-induced pro-survival transcription largely overlaps with that of lipopolysaccharide (LPS), a natural ligand of TLR4 (18). PXL activates TLR4 by binding to human or mouse MD2, an adaptor protein that confers LPS responsiveness of the TLR4 cascade (19). Second, PXL activates several pro-oncogenic signaling including NF-κB (20), MAPK (21) and PI3K (22). This effect of PXL has been shown in many human cancers including breast (20), prostate (23), ovarian (17), colon (24), lung, and pancreatic (25)tumors. Third, PXL increases expression of inflammatory cytokines in both tumor lines (12, 20, 26) and human cancer patients (14). Fourth,TLR4 is likely to transducer PXL signals because anti-TLR4 siRNA downregulates the expression of inflammatory cytokines induced by PXL (15, 26). Lastly, overexpressed TLR4 in human tumors correlates with resistance to PXL therapy (26), recurrence (27), clinical stage and grade (28), and poor patient survival (17). Taken together, this evidence suggests that TLR4 expression in human BC contributes to resistance to PXL therapy due to transcriptional upregulation of pro-survival genes.

To test this hypothesis, we determined TLR4 expression in a broad panel of BC lines, correlated cell responsiveness to PXL with TLR4 expression, and generated two isogenic lines with either reduced or increased TLR4 expression. These new BC models enabled us to analyze the role of TLR4 in PXL resistance using a variety of *in vitro* and *in vivo* approaches. The results strongly suggest that TLR4-mediated increase of cytokines and their receptors protects tumors from chemotherapy through activation of autocrine loops in neoplastic cells that may, in turn, activate paracrine pathways in the tumor environment.

Materials and Methods

Materials

LPS derived from Escherichia coli 055:B5, TRI-reagent, and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and supplements were from Lonza (Basel, Switzerland). SB225002 and PD98059 were purchased from EMD Millipore (Billerica, MA).

Plasmids and study drugs

Human TLR4 CDS ligated into pCDNA3.1 plasmid (TLR4-pCDNA3.1) was purchased from Addgene (Cambridge, MA). TLR4 shRNA plasmid (TLR4-psiRNA) was purchased from InvivoGen (San Diego, CA). The pKT2-IRES-PURO plasmid was a generous gift from Dr. Wilber (Southern Illinois University, IL, USA). Paclitaxel albumin-bound nanoparticles (nab-PXL) was obtained from Abraxis BioScience (now Celgene), Los Angeles, CA. Paclitaxel (Taxol®) was obtained from Bristol-Myers Squibb, NY.

Antibodies

Primary antibodies were: goat anti-hTLR4 (Imgenex, CA); rabbit anti-Bcl-2, anti-Bcl-xL, anti-p-Akt and anti-Akt (Cell Signaling, MA); rabbit anti-p65, anti-p50 and anti-p-p50 (Santa Cruz Biotechnology, CA); rabbit anti-p-p65 (Epitomics, CA), rabbit anti-active caspase-3 (R&D Systems, MN), and mouse anti-β-actin (JLA20) (Developmental Studies Hybridoma Bank, IA). Secondary HRP-conjugated anti-goat, anti-rabbit, and anti-mouse antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Human BC tissues and cell lines

Human tumor and normal BC tissues were obtained from ILSBio (Chestertown, MD). Luciferase-tagged MDA-MB-231 and HCC1806 cells were cultured in 10% DMEM with standard additives at 37°C in 10% CO_2. All cell lines were authenticated by ATCC and tested for mycoplasma using a kit from Roche Diagnostics GmbH (Penzberg, Germany).

Generation of stable sublines with modified TLR4

To suppress TLR4 expression, MDA-MB-231 cells were stably transfected with TLR4-psiRNA or scrambled shRNA followed by selection with zeocin (30μg/ml). To overexpress TLR4, HCC1806 line was transfected with human TLR4 CDS subcloned in pKT2-IRES-PURO vector or empty plasmid followed by selection with puromycin (1μg/ml). Changes in TLR4 expression were determined by RT-qPCR and Western blot. Modified sublines were designated as 231^{Cntrl} and 231^{TLR4-} or 1806^{Cntrl} and 1806^{TLR4+} for MDA-MB-231 and HCC1806 sublines, respectively.

RT-PCR and RT-qPCR

RNA was extracted by TRI-reagent and reverse transcribed using Revert Aid cDNA synthesis kit (Fermentas, Canada). Primers were designed based on CDS of human targets found in NCBI database. All primer sequences are listed in supplementary Table S1. Targets were amplified for 35 cycles followed by gel analysis and imaging. For RT-qPCR, cells were treated with LPS (100ng/ml) or nab-PXL (10nM) followed by transcript analysis using Go TaqGreen Master Mix (Promega, WI) and Real-Time PCR machine (Applied BioSystems, CA). Data were normalized to β-actin and relative mRNA expression was determined using the ΔΔCt method (29).

Western blot analysis

Cells seeded in a 6-well plate at the density of 0.5×10^6 adhered overnight prior to treatment with 10nM nab-PXL for 0, 12, 24, and 48hrs followed by lysis in 150μl buffer containing 50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 1%Triton-X100, 0.1% SDS, and protease inhibitors. Lysates were cleared at 13,000rpm for 10min, boiled, separated on 12% SDS gels and transferred to a nitrocellulose membrane followed by overnight incubation with primary antibodies against TLR4, Bcl-2, Bcl-xL, NF-κB p50, p-p50, NF-κB p65, p-p65, Akt, p-Akt, ERK1/2, p-ERK1/2, or β-actin. Protein bands were visualized by a Fujifilm LAS-3000 camera after a 1hr-incubation with HRP-conjugated secondary antibodies and development with ECL (Pierce, IL).

Cytotoxic assay

Cells seeded at the density of 50,000 cells per well in 24-well plates were treated with 0–100nM of PXL, nab-PXL or plain medium. After 48hrs, viable cells were enumerated to determine the IC_{50}. Each condition was tested in duplicate and reproduced thrice.

FACS analysis of apoptosis and cell cycle

Apoptotic cells were visualized using Annexin V detection kit according to the manufacturer's instructions (BD Pharmingen, CA). Briefly, nab-PXL treated cells (10nM) for 48hrs were incubated with FITC conjugated-Annexin V and propidium iodide (PI). After a-15min incubation, samples were analyzed by a Becton Dickinson FACS Calibur flow cytometer. Cell cycle phase distribution was determined in 70% alcohol-fixed cells stained with 10μg/ml of PI, sorted by FACS and analyzed using the CellQuest software (Becton Dickinson).

Measurement of cytokine concentrations by ELISA

Conditioned medium (CM) was collected from MDA-MB-231 and HCC1806 lines treated with 10nM of nab-PXL or plain medium. Concentrations of IL-6, IL-8, and TNF-α were determined by ELISA kits purchased from Peprotech (Rocky Hill, NJ). All experiments were performed in duplicate and reproduced thrice. Results are presented as mean pg of cytokine ±S.D. normalized per 10^6 cells.

Animal studies

Tumor growth of orthotopically implanted cell lines was monitored as described previously (3, 4). Briefly, 4×10^6 cells suspended in 50% Matrigel were implanted into the mammary fat pad (MFP) of 4–6 week-old female SCID mice (Taconic, Hudson, NY). Every 2–3 days, perpendicular tumor diameters were measured by digital caliper and used to calculate tumor volume according to the formula: volume = $Dd^2\pi/6$, where D equals larger diameter and d equals smaller diameter. Animal care was in accordance with institutional guidelines.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (La Jolla, CA). Results are expressed as mean ±S.D. Combination Index (CI) indicating synergism of two drugs was calculated by CompuSyn software (CompuSyn Inc., NY). Statistical significance for continuous variables and categorical covariants was determined by Student's paired *t*-test and Chi-square test, respectively. P-values 0.05 were considered significant.

Results

TLR4 expression and function in human BC tissues and cell lines

We first determined the frequency of TLR4 expression in human BC clinical samples and established lines. RT-qPCR analysis of 20 malignant breast tumors and 5 normal mammary tissues showed significantly(P-value=0.01) upregulated TLR4 expression in invasive BC as compared with non-malignant breast (Fig. 1A). Screening of BC lines by endpoint and RT-qPCR showed that 11 out of 16 (68%) lines had moderate to high TLR4 expression (Figs. 1B and 1C). The MDA-MB-231 line had the highest expression (53-fold above the level in negative lines) whereas HCC1806 line did not expressTLR4. Western blot confirmed TLR4 protein expression in MDA-MB-231 and HCC1806 lines (Fig. 1B, insert). These two lines were selected to represent TLR4-positive and -negative BC.

We next determined the functionality of TLR4 in these lines. Cells were treated with 100ng/ml of LPS followed by quantitative analysis of TLR4 targets such as IL-6, IL-8, TNF-α and

MCP-1. LPS induced ~100-fold higher response in MDA-MB-231 as compared with HCC1806 cells. The effect in MDA-MB-231 cells was mediated specifically via TLR4 because94% of the response was suppressed by an anti-hTLR4 antibody (Fig. S1). In contrast, a minor response in HCC1806 cells was TLR4-independent suggesting that other TLRs might be responsible for this effect. Indeed, HCC1806 cells express functional TLR2 and TLR9 (Fig. S2) that might weakly respond to TLR4 ligands.

We next asked whether MDA-MB-231 and HCC1806 differentially respond to PXL, a mimetic of LPS (30, 31). Cells were treated for 48hrs with nab-PXL followed by RT-qPCR analysis of IL-6, IL-8, TNF-α and MCP-1 mRNA. As shown in Fig. 1D, TLR4$^+$ MDA-MB-231 cells responded to PXL in a dose-dependent manner with robust upregulation of cytokines ranging from a 6.5-fold for IL-8 (<0.01) to 9-fold for TNF-α (<0.05). This effect was mediated by TLR4 as indicated by a 95% reduction in cytokine expression in the presence of anti-hTLR4 antibody (Fig. 1E). Minor increase in cytokines was also noted in HCC1806 cells, albeit this response was not affected by anti-hTLR4 antibody (Figs. 1F, 1G).

TLR4 expression in BC lines correlates with resistance to PXL

Functional TLR4 may significantly enhance inflammation in the tumor environment leading to increased chemoresistance (32, 33), and metastasis(34). To test whether PXL-induced inflammatory responses protect cells from chemotherapy, we measured IC$_{50}$ of PXL in TLR4$^+$ MDA-MB-231 and TLR4-negative HCC1806 cells. As shown in Figs. 2A and 2B, TLR4$^+$ MDA-MB-231, was 4-fold more resistant to PXL as compared with HCC1806 (IC$_{50}$ of 12.5 *vs.*3.25nM; *P*-value 0.003). This difference was equally observed in PXL and nab-PXL treated cells indicating that this effect is formulation-independent. We also tested four additional lines with either high or low TLR4 expression. As shown in supplementary Table S2,the mean IC$_{50}$ for lines enriched withTLR4 was 2-fold higher (*P*-value=0.037) than for those with low level of this receptor. This finding supported the role of TLR4 in chemoresistance although the firm conclusion could be reached only by comparing genetically identical lines.

To this end, we generated and functionally characterized isogenic derivatives of MDA-MB-231 and HCC1806 with differential TLR4 expression. Compared with controls, the selected MDA-MB-231 clones had 80% reduced TLR4 whereas HCC1806 clones had 10,000-fold higher TLR4 expression (Fig. S3). Modified TLR4 expression corresponded to changes in both LPS and PXL-induced upregulation of inflammatory cytokines. Downstream TLR4 mRNA and protein targets were significantly (50–60%) reduced in 231^{TLR4-} and 5–25-fold upregulated in 1806^{TLR4+} lines treated with TLR4 ligands. These clones designated **231^{TLR4-}** or **1806^{TLR4+}**,along with their controls **231^{Cntrl}** and **1806^{Cntrl}**,were used to analyze the TLR4 role in PXL resistance.

After validating functionality of an ectopically expressed TLR4, we used the modified lines to determine differences in cytotoxic response to PXL. Figs. 2C and 2D show that 231^{TLR4-} cells were more sensitive to nab-PXL (2.5 fold; P<0.04) than 231^{Cntrl}cells, whereas1806^{TLR4+} line was nearly 3-fold more resistant to PXLthan1806^{Cntrl} cells. In addition to genetic manipulation, the role of TLR4 in conferring resistance to PXL was also demonstrated by a monospecific anti-hTLR4 antibody (Fig. 2E), specific intracellular and extracellular TLR4 inhibitors, TAK-242 and LPS-EK^{ultra} (Figs. 2F, 2G). These structurally unrelated agents targeting TLR4 share the capacity to increase response to PXL by 2.0 to 2.5 fold. Collectively, these data show that TLR4 expressed in human tumor cells regulates both expression of inflammatory cytokines and sensitivity to PXL.

TLR4 promotes survival of PXL-treated tumor cells by increasing levels of anti-apoptotic proteins

Increased chemoresistance typically correlates with upregulation of pro-survival proteins (17, 26, 32). We therefore hypothesized that TLR4 promotes survival of PXL-treated cells by activating NF-κB pathway known to transcribe pro-survival genes (35, 36). To test this hypothesis, cells were treated with 10nM of nab-PXL for 0–48hrs followed by Western blot analysis of phosphorylated and total p50, p65, Akt, Bcl-xL, and Bcl-2. Depletion of TLR4 in MDA-MB-231 substantially decreased expression of both phosphorylated and non-phosphorylated p50 and p65, as well as, p-Akt, Bcl-xL and Bcl-2 (Fig. 3A). Conversely, TLR4 overexpression in HCC1806 cells increased phosphorylation of NF-κB concomitant with upregulation of Bcl-2 and Bcl-xL (Fig. 3B).

We next examined the consequences of TLR4-dependent biochemical changes in major pro-survival proteins for developing resistance to PXL therapy. PXL induces apoptosis by over-stabilizing microtubules which leads to cell arrest in the G2/M phase (1). Therefore, we hypothesized that TLR4 might alter distribution of tumor cells through cell cycle phases. To test this hypothesis, cells were treated with nab-PXL (10nM) for 48hrs, stained with PI and analyzed for phase distribution by FACS. As shown in Fig. 3C, TLR4 knockdown significantly increased the number of cells arrested at the G2/M phase as evidenced by 58% of 231^{TLR4-} cells detected in this phase after PXL treatment as compared with 35% in the control line(P<0.05). An opposite distribution was observed in the HCC1806 model (65% in control vs. 34% in 1806^{TLR4+}, Figs. 3D and S4).

These results suggested that tumor cells with reduced TLR4 are more susceptible to PXL-induced apoptosis as compared with TLR4-overexpressing cells. To test this hypothesis, we quantified PXL-induced apoptosis in MDA-MB-231 and HCC1806 lines. Cells were treated as described above followed by quantification of apoptotic cells identified by double-staining with PI and Annexin V. Fig.3E shows that% of apoptotic cells significantly increased in 231^{TLR4-} as compared with 231^{Cntrl} (43% vs. 29%, P<0.05). An identical experiment in HCC1806 model independently confirmed causalitybetweenTLR4 and drug-induced apoptosis: only 28% of 1806^{TLR4+} cells were apoptotic as compared with 40% in 1806^{Cntrl} (Fig. 3F; P<0.05). Similar results were obtained by identifying apoptotic cells using anti-active caspase-3 antibody. As shown in Fig. S4, 43% of 231^{TLR4-} cells were positive for caspase-3 as compared with 20% in control line (2.2-fold increase, P<0.05). Similarly, 1806^{TLR4+} cells showed a 2.1-fold decreased apoptosis compared with 1806^{Cntrl} cells (P<0.05). These results suggest that PXL-mediated activation of the TLR4 pathway concordantly diminishes the number of cells arrested in G2/M phase and those undergoing apoptosis. This, in turn, may significantly increase tumor recurrence after cessation of therapy.

PXL-activated TLR4 upregulates both inflammatory ligands and receptors potentially creating pro-survival autocrine loops

In macrophages, the hallmark of LPS activated-TLR4 signaling is coincident induction of inflammatory cytokines and matching receptors. Generation of these autocrine loops amplifies intracellular pathways that enhance proliferation, migration, and survival of pathogen-fighting immune cells. Because PXL is an LPS mimetic(30), we hypothesized that a similar effect can be produced by taxanes on $TLR4^+$ cancer cells. To test this hypothesis, we compared the expression of both cytokines and matching receptors in control and PXL-treated MDA-MB-231 and HCC1806 cell lines. Cells were treated with nab-PXL followed by RT-qPCR analysis of ~100 inflammatory cytokines and matching receptors. Using this approach, we identified a substantial number of targets upregulated by nab-PXL by at least 1.5 fold (Fig. 4A). 1806^{Cntrl} line lacking TLR4 was the least responsive line with 17.9% of

upregulated receptors whereas the broadest responses (50% upregulated targets) were obtained in the 231^{Cntrl} cells that have the highest expression of TLR4. This response was TLR4-dependent because both the number of targets and the degree of their upregulation correlated with TLR4 expression in both MDA-MB-231 and HCC1806 models(Figs. 4A, 4B). Importantly, some targets became detectable only after exposure to PXL(Tables S3, S4) suggesting that this therapy not only intensifies inflammation, but also qualitatively changes the tumor environment.

Significantly, several upregulated cytokines in both BC models were co-induced with matching receptors, potentially creating autocrine growth-promoting loops (Figs. 4A, 4C). This is best illustrated by the changes in HCC1806 line in which ectopic expression of TLR4 increased the number of matching pairs by 328% (16.6% and 54.5% in 1806^{Cntrl} and 1806^{TLR4+}, respectively; Fig. 4A). Using this profiling, we identified several new ligand-receptor pairs induced by TLR4 as indicated by significant differences between the control and TLR4-manipulated lines(Fig. 4C, P<0.05). In the 231^{Cntrl} line, the highest upregulated pairswere: CXCL2•CXCR2 (11.40 ± 1.89 and 9.43 ± 1.60 fold increase for ligand and receptor, respectively), CCL20•CCR6 (9.95 ± 1.53 and 8.02 ± 0.62 fold), and IL-8•IL-8R (7.46 ± 0.55 and 6.76 ± 1.93 fold). Pairs upregulated in 1806^{TLR4+} cells included CSF1•CSFR1, BAFF•BAFFR and INHBB•ACVR2B. This study, therefore, suggests that treatment with nab-PXL creates a broad inflammatory response that fundamentally alters the tumor milieu, and consequently, the response to therapy.

PXL-activated cytokine-receptor autocrine loops contribute to drug resistance through increased phosphorylation of AKT and ERK1/2

We next examined whether drug-upregulated cytokine-receptor pairs contribute to chemoresistance. We tested whether neutralizing antibodies or specific pharmacological inhibitors of cytokines or receptors increase tumor cell sensitivity to PXL. Out of 7 most upregulated pairs in MDA-MB-231 (Fig. 4C), we assessed the effect of interrupting a putative autocrine loop for 5 pairs (IL-6, IL-8, MCP-1, CXCL1 and CXCL2). While neutralization of IL-6 or IL-8 had no significant effect on nab-PXL toxicity, blockade of CCR4 and CXCR2 receptors was highly synergistic with PXL treatment (Figs. 5A, 5B). Analysis of synergism of blocking CCR4 and CXCR2 by CompuSyn software yielded highly significant Combination Index (CI) values for both inhibitors of 0.59 and 0.52, respectively (Fig. 5C). Both pathways activate MEK that phosphorylates ERK1/2(37). Indeed, combination of MEK inhibitor PD98059 (100μM) with nab-PXL was highly synergistic (CI=0.45) resulting in 69% inhibition compared with ~50% caused by nab-PXL alone (Figs. 5C and 5D). Synergy with nab-PXL was also evident by enhanced phosphorylation of ERK1/2 and AKT (Fig. 5E). Collectively, these results indicate that CXCL1/2•CXCR2 and MCP-1•CCR4 loops functionally contribute to survival of tumor cells during chemotherapy.

Expression of TLR4 in BC cells dictates their responses to PXL therapy *in vivo*

Substantial differences in susceptibility of BC lines to PXL *in vitro* suggested a prominent role of TLR4 in chemoresistance *in vivo*. To test this hypothesis, we measured tumor growth rate of orthotopically implanted lines with differential TLR4 expression. All 6 tested clones of 231^{TLR4-} grew substantially slower than control 231^{Cntrl} line (Fig. 6A). The period prior to exponential tumor growth of 231^{TLR4-} clones doubled in most mice with some requiring>110 days compared with only 10–15 days in control mice (Fig. 6B). Expectantly, all 1806^{TLR4+} clones (n=4) grew much faster than control lines (P>0.02; Fig. 6C).

Treatment with nab-PXL (10mg/kg) produced drastically different results in isogenic lines with modified TLR4 expression (Figs. 6D–F). Within few weeks after PXL treatment, 100%

of control mice bearing MDA-MB-231 tumors had recurrence. In sharp contrast, only 1 out of 6 mice (17%) with 231^{TLR4-} tumors had recurrence 127 days post-treatment while rest of the group was disease-free for experimental duration of 6 months ($P>0.0001$ *vs.* control, Fig. 6D). Consistently, 100% of mice with 1806^{TLR4+} clones recurred immediately upon cessation of therapy whereas 40% of mice with tumors lacking TLR4 had no palpable masses after chemotherapy(Fig. 6F). The mean inhibition in the latter group was 95% as compared with 10% inhibition in TLR4-overexpressing group ($P>0.001$, Fig. 6F). These results unequivocally show that TLR4 plays a major role in determining the response of tumor cells to PXL therapy.

Discussion

TLR4 has been previously shown to be overexpressed in a variety of human cancers (17, 38) and to correlate with tumor progression (39), recurrence (27), metastasis (38, 40), and resistance to PXL therapy (17, 26). However, a pro-tumorigenic effect of TLR4 was mainly demonstrated using LPS (25, 41), a bacterial component that is absent in epithelial malignancies outside of the gastrointestinal tract. More relevant to cancer research is use of PXL, an LPS mimetic (42)and a widely-administrated chemotherapeutic drug(43). PXL-mediated resistance to therapy has been previously shown in ovarian cancer cells *in vitro*(15, 26). Here, we present extensive evidence demonstrating the chemoresistance-promoting role of TLR4 in breast cancer using both *in vitro* and *in vivo* models. We show that TLR4 is overexpressed in clinical BC, and it is specifically responsible for mediating chemoresistance to PXL as evident in increasing IC_{50} in tumor cells *in vitro* and causing recurrence in BC *in vivo*.

TLR4 is considered as a "double-edge sword" with some studies showing its contribution to chemoresistance (17) and others arguing its role in enhancing anti-cancer immunity (44, 45). Here, we show in two distinct BC models that activatedTLR4 pathway drastically diminishes therapeutic efficacy as evidenced by reduced apoptosis of treated cells *in vitro* and prominent recurrence of treated tumors *in vivo*. This effect is mediated by NF-κB-mediated transcription of inflammatory and pro-survival genes (Figs. 3, 4). These findings indicate that TLR4 affords tumor evasion from PXL therapy rather than enhancing its cytotoxic effect.

While this conclusion is consistent with the evidence from ovarian cancer models (17, 26, 32), our study demonstrates several new findings underscoring the functional significance of TLR4 in BC. We show that TLR4 is broadly expressed in clinical BC as well as the in majority (68%) of examined BC lines (Fig. 1). We also show that TLR4 is functional in human cancer cells as evidenced by upregulated cytokine expression after treatment with either LPS or PXL(Fig. 1). Previous reports suggested that only mouse TLR4-MD2 complex transduces PXL signaling (18, 19). In contrast, we found that both PXL and nab-PXL phosphorylates NF-κB in $TLR4^+$ human cancer cells followed by up to 15-fold upregulation of multiple targets induced in dose- and time-dependent manners. These data indicate that despite the biochemical difference between human and mouse TLR4-MD2 complexes (42), PXL can enhance chemoresistance of $TLR4^+$ breast tumors in a clinical setting.

The specificity of TLR4 in mediating tumor-promoting PXL effects is shown here by genetic manipulation of TLR4 in two BC lines and by reduced cytotoxicity and cytokine expression following treatment with a monospecific anti-hTLR4 antibody, an extracellular LPS lipid antagonist, and an intracellular inhibitor cyclohexene TAK-242. The fact that several structurally unrelated TLR4 inhibitors significantly increase sensitivity of tumor cells to PXL strongly argues for important role of this receptor in conferring resistance. This is also supported by the evidence that PXL promoted resistance in $TLR4^+$ cells regardless of

their genetic background (Table S2) suggesting that TLR4 expression could be an independent prognostic marker for resistance to therapy.

Mechanistically, TLR4-dependent chemoresistance could be mediated by pro-survival autocrine loops some of which have been identified in this study (Tables S3, S4). Although PXL-mediated increases in some cytokines have been previously reported in both human BC patients (14, 46)and experimental BC models (3, 4), this is the first study that analyzed co-expression of inflammatory ligands and receptors in a TLR4-dependent manner. Two TLR4-regulated pathways that seem to be important in this regard are MCP-1•CCR4 and CXCL−1/−2•CXCR1/2. This is consistent with large body of evidence that shows strong correlations between MCP-1(47), CCR4(48), CXCL−1/−2(49), and CXCR1/2(50) with chemoresistance and metastasis. Importantly, *in vivo* cytokine-activated pathways have the potential to promote tumor cell survival through both autocrine and paracrine loops. This could explain the discrepancy between 2–3 fold shift in IC_{50} to nab-PXL in tumor cells *in vitro*(Fig. 2) and drastic 83% reduction in recurrence observed in TLR4-depleted tumors *in vivo* (Fig. 6). This evidence strongly suggests that therapy-induced activation of TLR4 pathway promotes tumor cell survival not only through autocrine loops, but also by activating secondary pathways in host cells in the tumor environment.

In summary, we show that TLR4 increases resistance to PXL in human BC cells by activating the NF-κB pathway leading to transcription of inflammatory genes that alter the tumor environment through autocrine and paracrine loops. TLR4 signaling in tumor cells may significantly reduce therapeutic efficacy by promoting chronic inflammation, angiogenesis, and recovery of damaged cells. Importantly, this pathway may also promote metastasis. This study, therefore, suggests that blocking TLR4 signaling during PXL therapy is essential for increasing the responsiveness of primary tumors and preventing relapse at secondary sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

List of Abbreviations

BC	breast cancer
CDS	complementary DNA sequence
DMEM	Dulbecco's modified Eagle's medium
HRP	horseradish peroxidase
FACS	fluorescence-activated cell sorter
IC_{50}	inhibitory concentration-50
IL-6	interleukin 6
IL-8	interleukin 8
LPS	lipopolysaccharide

| MCP-1 | monocyte chemoattractant protein-1 |
NF-κB	nuclear factor kappa-light-chain-enhancer
PBS	phosphate buffer saline
PI3K	phosphatidylinositol-3-kinase
PXL	paclitaxel
nab-PXL	nanoparticle albumin-bound paclitaxel
RT-(q)PCR	reverse transcription (quantitative)polymerase chain reaction;TLR4 -Toll like receptor-4
TNFα	tumor necrosis factor alpha
SEM	standard error of the mean
VEGF-A	Vascular Endothelial Growth Factor-A

References

1. McGrogan BT, Gilmartin B, Carney DN, McCann A. Taxanes, microtubules and chemoresistant breast cancer. Biochim Biophys Acta. 2008; 1785:96–132. [PubMed: 18068131]

2. Berrieman HK, Lind MJ, Cawkwell L. Do beta-tubulin mutations have a role in resistance to chemotherapy? Lancet Oncol. 2004; 5:158–164. [PubMed: 15003198]

3. Volk LD, Flister MJ, Bivens CM, Stutzman A, Desai N, Trieu V, et al. Nab-paclitaxel efficacy in the orthotopic model of human breast cancer is significantly enhanced by concurrent anti-vascular endothelial growth factor A therapy. Neoplasia. 2008; 10:613–623. [PubMed: 18516298]

4. Volk LD, Flister MJ, Chihade D, Desai N, Trieu V, Ran S. Synergy of nab-paclitaxel and bevacizumab in eradicating large orthotopic breast tumors and preexisting metastases. Neoplasia. 2011; 13:327–338. [PubMed: 21472137]

5. Grosjean J, Kiriakidis S, Reilly K, Feldmann M, Paleolog E. Vascular endothelial growth factor signalling in endothelial cell survival: a role for NFkappaB. Biochem Biophys Res Commun. 2006; 340:984–994. [PubMed: 16410078]

6. Rowinsky EK. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med. 1997; 48:353–374. [PubMed: 9046968]

7. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly in vitro by taxol. Nature. 1979; 277:665–667. [PubMed: 423966]

8. Horwitz SB. Taxol (paclitaxel): mechanisms of action. Ann Oncol. 1994; 5(Suppl 6):S3–S6. [PubMed: 7865431]

9. Damascelli B, Cantu G, Mattavelli F, Tamplenizza P, Bidoli P, Leo E, et al. Intraarterial chemotherapy with polyoxyethylated castor oil free paclitaxel, incorporated in albumin nanoparticles (ABI-007): Phase II study of patients with squamous cell carcinoma of the head and neck and anal canal: preliminary evidence of clinical activity. Cancer. 2001; 92:2592–2602. [PubMed: 11745194]

10. Gradishar WJ, Tjulandin S, Davidson N, Shaw H, Desai N, Bhar P, et al. Phase III trial of nanoparticle albumin-bound paclitaxel compared with polyethylated castor oil-based paclitaxel in women with breast cancer. J Clin Oncol. 2005; 23:7794–7803. [PubMed: 16172456]

11. Kirikae T, Ojima I, Kirikae F, Ma Z, Kuduk SD, Slater JC, et al. Structural requirements of taxoids for nitric oxide and tumor necrosis factor production by murine macrophages. Biochem Biophys Res Commun. 1996; 227:227–235. [PubMed: 8858130]

12. White CM, Martin BK, Lee LF, Haskill JS, Ting JP. Effects of paclitaxel on cytokine synthesis by unprimed human monocytes, T lymphocytes, and breast cancer cells. Cancer Immunol Immunother. 1998; 46:104–112. [PubMed: 9558006]

13. Lee LF, Haskill JS, Mukaida N, Matsushima K, Ting JP. Identification of tumor-specific paclitaxel (Taxol)-responsive regulatory elements in the interleukin-8 promoter. Mol Cell Biol. 1997; 17:5097–5105. [PubMed: 9271387]

14. Pusztai L, Mendoza TR, Reuben JM, Martinez MM, Willey JS, Lara J, et al. Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy. Cytokine. 2004; 25:94–102. [PubMed: 14698135]

15. Wang AC, Su QB, Wu FX, Zhang XL, Liu PS. Role of TLR4 for paclitaxel chemotherapy in human epithelial ovarian cancer cells. Eur J Clin Invest. 2009; 39:157–164. [PubMed: 19200169]

16. Aggarwal BB, Shishodia S, Takada Y, Banerjee S, Newman RA, Bueso-Ramos CE, et al. Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. Clin Cancer Res. 2005; 11:7490–7498. [PubMed: 16243823]

17. Silasi DA, Alvero AB, Illuzzi J, Kelly M, Chen R, Fu HH, et al. MyD88 predicts chemoresistance to paclitaxel in epithelial ovarian cancer. Yale J Biol Med. 2006; 79:153–163. [PubMed: 17940625]

18. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K, Nishijima M. Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem. 2000; 275:2251–2254. [PubMed: 10644670]

19. Zimmer SM, Liu J, Clayton JL, Stephens DS, Snyder JP. Paclitaxel binding to human and murine MD-2. J Biol Chem. 2008; 283:27916–27926. [PubMed: 18650420]

20. Zaks-Zilberman M, Zaks TZ, Vogel SN. Induction of proinflammatory and chemokine genes by lipopolysaccharide and paclitaxel (Taxol) in murine and human breast cancer cell lines. Cytokine. 2001; 15:156–165. [PubMed: 11554785]

21. Small GW, Shi YY, Higgins LS, Orlowski RZ. Mitogen-activated protein kinase phosphatase-1 is a mediator of breast cancer chemoresistance. Cancer Res. 2007; 67:4459–4466. [PubMed: 17483361]

22. Wang Y, Qu Y, Niu XL, Sun WJ, Zhang XL, Li LZ. Autocrine production of interleukin-8 confers cisplatin and paclitaxel resistance in ovarian cancer cells. Cytokine. 2011; 56:365–375. [PubMed: 21742513]

23. Pei Z, Lin D, Song X, Li H, Yao H. TLR4 signaling promotes the expression of VEGF and TGFbeta1 in human prostate epithelial PC3 cells induced by lipopolysaccharide. Cell Immunol. 2008; 254:20–27. [PubMed: 18649875]

24. Tang XY, Zhu YQ, Wei B, Wang H. Expression and functional research of TLR4 in human colon carcinoma. Am J Med Sci. 2010; 339:319–326. [PubMed: 20228668]

25. Ikebe M, Kitaura Y, Nakamura M, Tanaka H, Yamasaki A, Nagai S, et al. Lipopolysaccharide (LPS) increases the invasive ability of pancreatic cancer cells through the TLR4/MyD88 signaling pathway. J Surg Oncol. 2009; 100:725–732. [PubMed: 19722233]

26. Szajnik M, Szczepanski MJ, Czystowska M, Elishaev E, Mandapathil M, Nowak-Markwitz E, et al. TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. Oncogene. 2009; 28:4353–4363. [PubMed: 19826413]

27. Gonzalez-Reyes S, Fernandez JM, Gonzalez LO, Aguirre A, Suarez A, Gonzalez JM, et al. Study of TLR3, TLR4, and TLR9 in prostate carcinomas and their association with biochemical recurrence. Cancer Immunol Immunother. 2010; 60:217–7726. [PubMed: 20978888]

28. Zhang JJ, Wu HS, Wang L, Tian Y, Zhang JH, Wu HL. Expression and significance of TLR4 and HIF-1alpha in pancreatic ductal adenocarcinoma. World J Gastroenterol. 2010; 16:2881–2888. [PubMed: 20556833]

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–408. [PubMed: 11846609]

30. Byrd-Leifer CA, Block EF, Takeda K, Akira S, Ding A. The role of MyD88 and TLR4 in the LPS-mimetic activity of Taxol. Eur J Immunol. 2001; 31:2448–2457. [PubMed: 11500829]

31. Ganta S, Amiji M. Coadministration of Paclitaxel and curcumin in nanoemulsion formulations to overcome multidrug resistance in tumor cells. Mol Pharm. 2009; 6:928–939. [PubMed: 19278222]

32. Kelly MG, Alvero AB, Chen R, Silasi DA, Abrahams VM, Chan S, et al. TLR-4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. Cancer Res. 2006; 66:3859–3868. [PubMed: 16585214]

33. Chen R, Alvero AB, Silasi DA, Mor G. Inflammation, cancer and chemoresistance: taking advantage of the toll-like receptor signaling pathway. Am J Reprod Immunol. 2007; 57:93–107. [PubMed: 17217363]

34. Finger EC, Giaccia AJ. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. Cancer Metastasis Rev. 2010; 29:285–293. [PubMed: 20393783]

35. Doan HQ, Bowen KA, Jackson LA, Evers BM. Toll-like receptor 4 activation increases Akt phosphorylation in colon cancer cells. Anticancer Res. 2009; 29:2473–2478. [PubMed: 19596916]

36. Zhang Y, Wang Y, Yuan J, Qin W, Liu F, Wang F, et al. Toll-like receptor 4 ligation confers chemoresistance to docetaxel on PC-3 human prostate cancer cells. Cell Biol Toxicol. 2012; 28:269–277. [PubMed: 22648782]

37. Fukazawa H, Noguchi K, Murakami Y, Uehara Y. Mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitors restore anoikis sensitivity in human breast cancer cell lines with a constitutively activated extracellular-regulated kinase (ERK) pathway. Mol Cancer Ther. 2002; 1:303–309. [PubMed: 12489846]

38. Gonzalez-Reyes S, Marin L, Gonzalez L, Gonzalez LO, Del Casar JM, Lamelas ML, et al. Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis. BMC Cancer. 2010; 10:665. [PubMed: 21129170]

39. Lee CH, Wu CL, Shiau AL. Toll-like receptor 4 signaling promotes tumor growth. J Immunother. 2010; 33:73–82. [PubMed: 19952954]

40. Liao SJ, Zhou YH, Yuan Y, Li D, Wu FH, Wang Q, et al. Triggering of Toll-like receptor 4 on metastatic breast cancer cells promotes alphavbeta3-mediated adhesion and invasive migration. Breast Cancer Res Treat. 2011; 133:853–863. [PubMed: 22042369]

41. Gonzalez-Reyes S, Fernandez JM, Gonzalez LO, et al. Study of TLR3, TLR4, and TLR9 in prostate carcinomas and their association with biochemical recurrence. Cancer Immunol Immunother. 2010; 60:217–226. [PubMed: 20978888]

42. Kawasaki K, Nogawa H, Nishijima M. Identification of mouse MD-2 residues important for forming the cell surface TLR4-MD-2 complex recognized by anti-TLR4-MD-2 antibodies, and for conferring LPS and taxol responsiveness on mouse TLR4 by alanine-scanning mutagenesis. J Immunol. 2003; 170:413–420. [PubMed: 12496426]

43. Wick N, Saharinen P, Saharinen J, Gurnhofer E, Steiner CW, Raab I, et al. Transcriptomal comparison of human dermal lymphatic endothelial cells ex vivo and in vitro. Physiol Genomics. 2007; 28:179–192. [PubMed: 17234577]

44. Molavi O, Ma Z, Hamdy S, Lavasanifar A, Samuel J. Immunomodulatory and Anticancer Effects of Intra-Tumoral Co-Delivery of Synthetic Lipid A Adjuvant and STAT3 Inhibitor, JSI-124. Immunopharmacol Immunotoxicol. 2008; 31:214–221. [PubMed: 18798092]

45. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007; 13:1050–1059. [PubMed: 17704786]

46. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boulamatsis D. Immune changes in patients with advanced breast cancer undergoing chemotherapy with taxanes. Br J Cancer. 2002; 87:21–27. [PubMed: 12085250]

47. Egunsola AT, Zawislak CL, Akuffo AA, Chalmers SA, Ewer JC, Vail CM, et al. Growth, metastasis, and expression of CCL2 and CCL5 by murine mammary carcinomas are dependent upon Myd88. Cell Immunol. 2012; 272:220–229. [PubMed: 22088941]

48. Li JY, Ou ZL, Yu SJ, Gu XL, Yang C, Chen AX, et al. The chemokine receptor CCR4 promotes tumor growth and lung metastasis in breast cancer. Breast Cancer Res Treat. 2012; 131:837–848. [PubMed: 21479551]

49. Acharyya S, Oskarsson T, Vanharanta S, Malladi S, Kim J, Morris PG, et al. A CXCL1 paracrine network links cancer chemoresistance and metastasis. Cell. 2012; 150:165–178. [PubMed: 22770218]

50. Singh S, Sadanandam A, Nannuru KC, Varney ML, Mayer-Ezell R, Bond R, et al. Small-molecule antagonists for CXCR2 and CXCR1 inhibit human melanoma growth by decreasing tumor cell proliferation, survival, and angiogenesis. Clin Cancer Res. 2009; 15:2380–2386. [PubMed: 19293256]
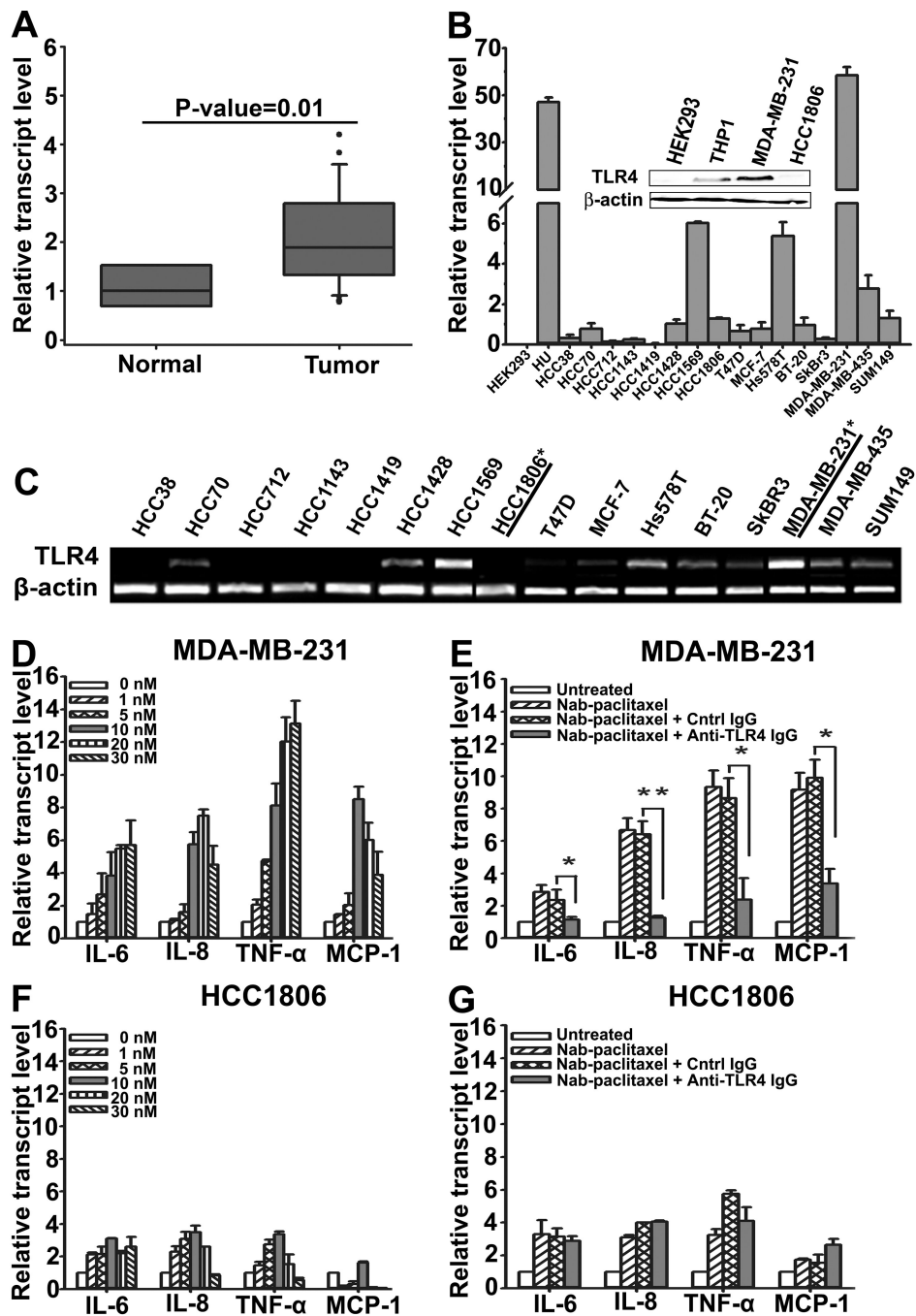
Figure 1. Frequency of TLR4 expression in human BC tumors and lines
(**A**) Analysis of TLR4 mRNA in human normal breast (n=5) and tumors (n=20) by RT-qPCR. (**B**) RT-qPCR analysis of TLR4 mRNA in BC lines. **Insert:** Western blot of TLR4 protein expression in MDA-MB-231 and HCC1806 cells. HEK293 and THP1 represent lines with negative and positive TLR4 expression, respectively. (**C**) RT-PCR analysis of TLR4 mRNA expression in cell lines. Underline and asterisk indicates the cell lines to represent TLR4$^+$ and TLR4$^-$ lines. (**D, F**) Activation of TLR4 pathway by paclitaxel inMDA-MB-231 and HCC1806 cells. RT-qPCR quantification of IL-6, IL-8, TNFα and MCP-1 in cells treated with nab-PXL (0–30nM) for 48hrs. (**E, G**) MDA-MB-231 and

HCC1806 cells were pre-treated with control-IgG or anti-TLR4 antibody (4ug/ml) for 2hrs before nab-PXL treatment (10nM) and analyzed as in 1D. Data are presented as β-actin normalized mRNA expression ±S.D. from two experiments done in duplicate. The P-values represent * <0.05 and ** <0.01 vs. control as determined by Student's paired *t* test.
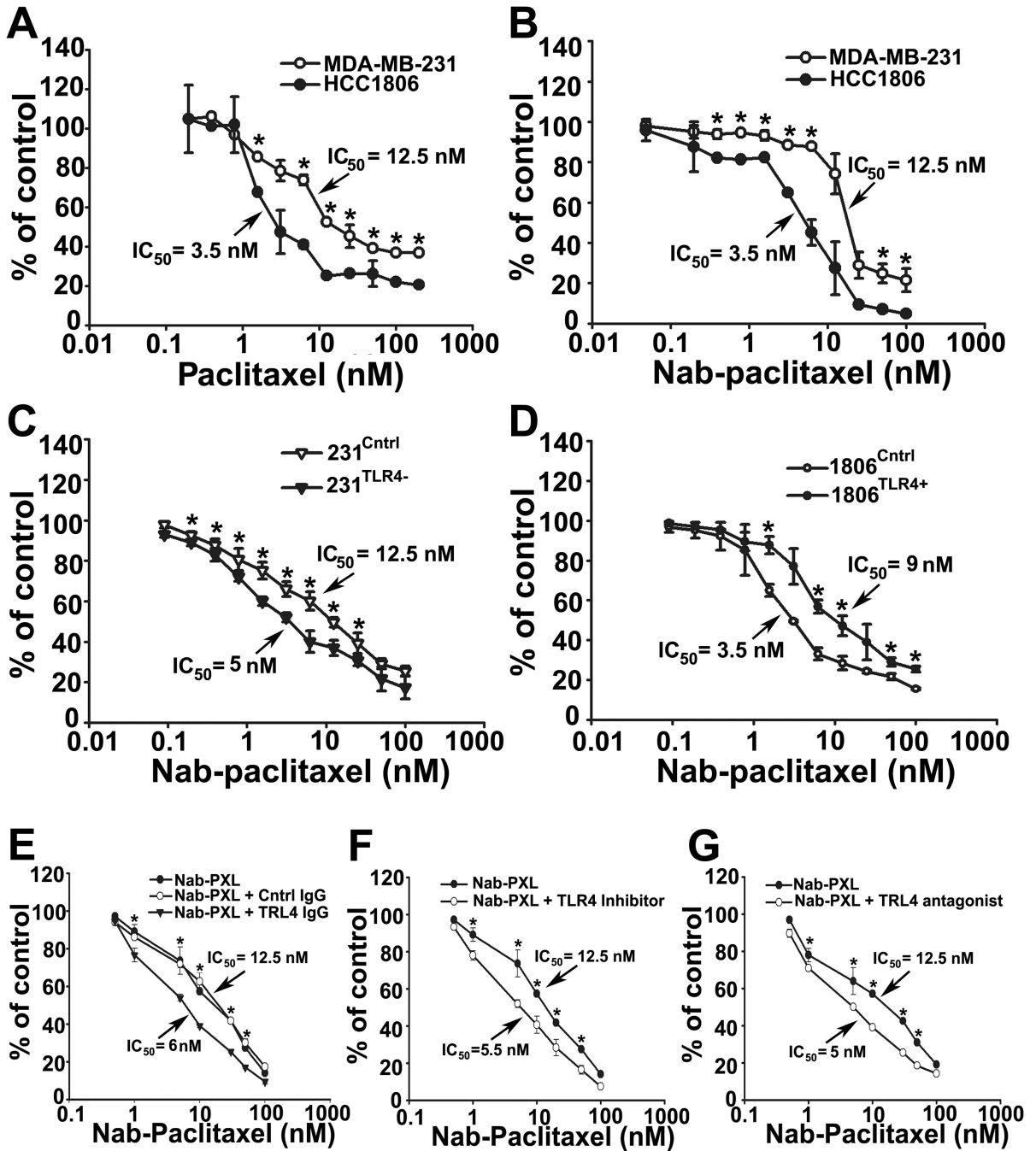
**Figure 2. TLR4-positive and negative lines significantly differ in sensitivity to PXL**
MDA-MB-231 and HCC1806 cells were treated with PXL **(A)** or nab-PXL **(B)** at indicated concentrations (0–100nM) for 48hrs followed by calculating IC50. **(C, D)** 231[Cntrl], 231[TLR4–] cells, 1806[Cntrl] and 1806[TLR4+] cells were treated and analyzed as described under 2A. **(E)** MDA-MB-231 cells were pretreated with 4μg/ml of anti-TLR4 or control IgG for 2hrs followed by analysis described under 2A. **(F, G)** Cells were pretreated with a TLR4 inhibitor TAK-242 (10uM) or with an LPS antagonist LPS-EK[Ultra] for 2hrs followed by analysis described under 2A. Data are presented as percent of viable cells ±S.D. from three

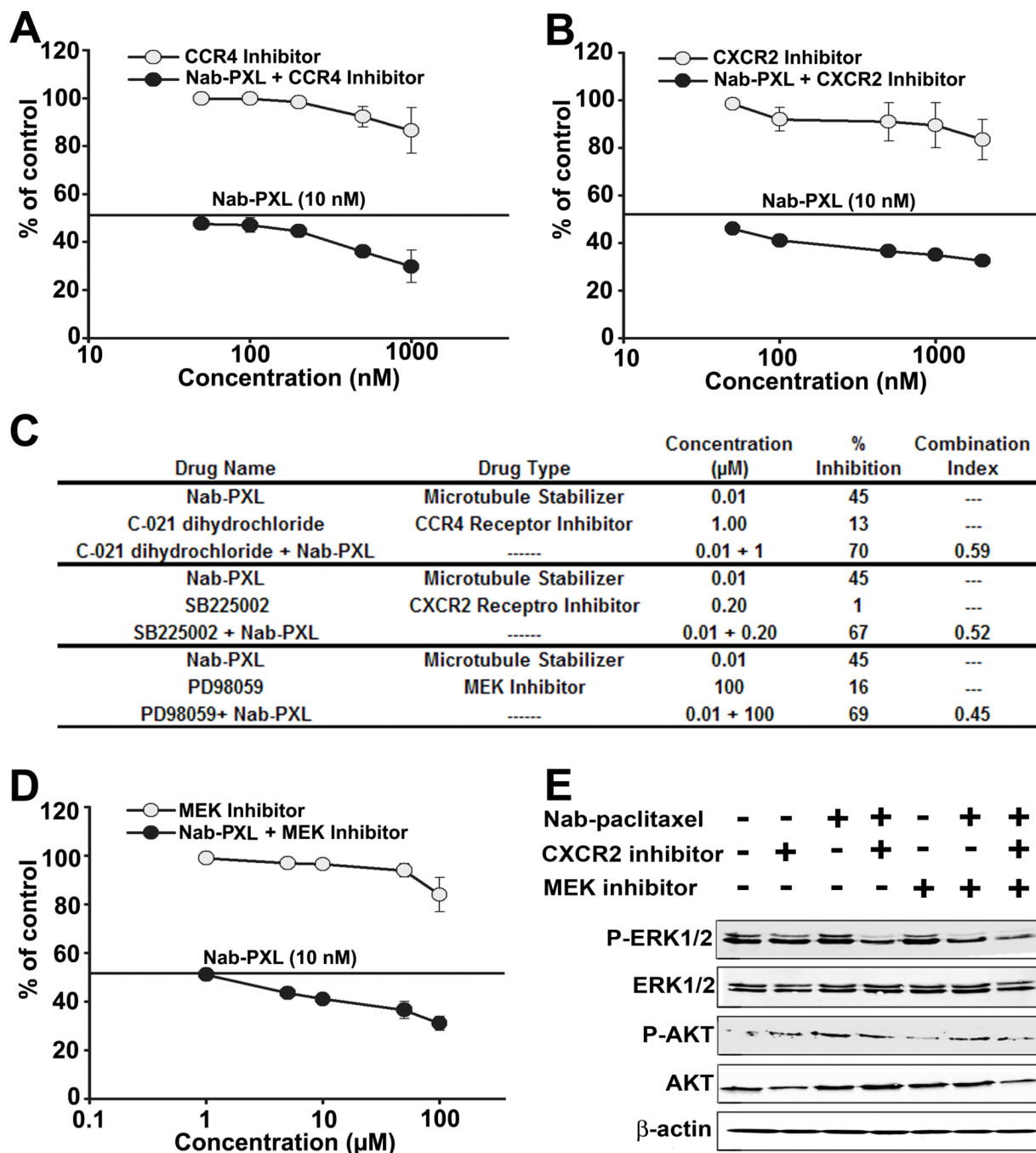experiments done in triplicate. The P-values represent * <0.05 vs. control as determined by Student's paired *t*-test.
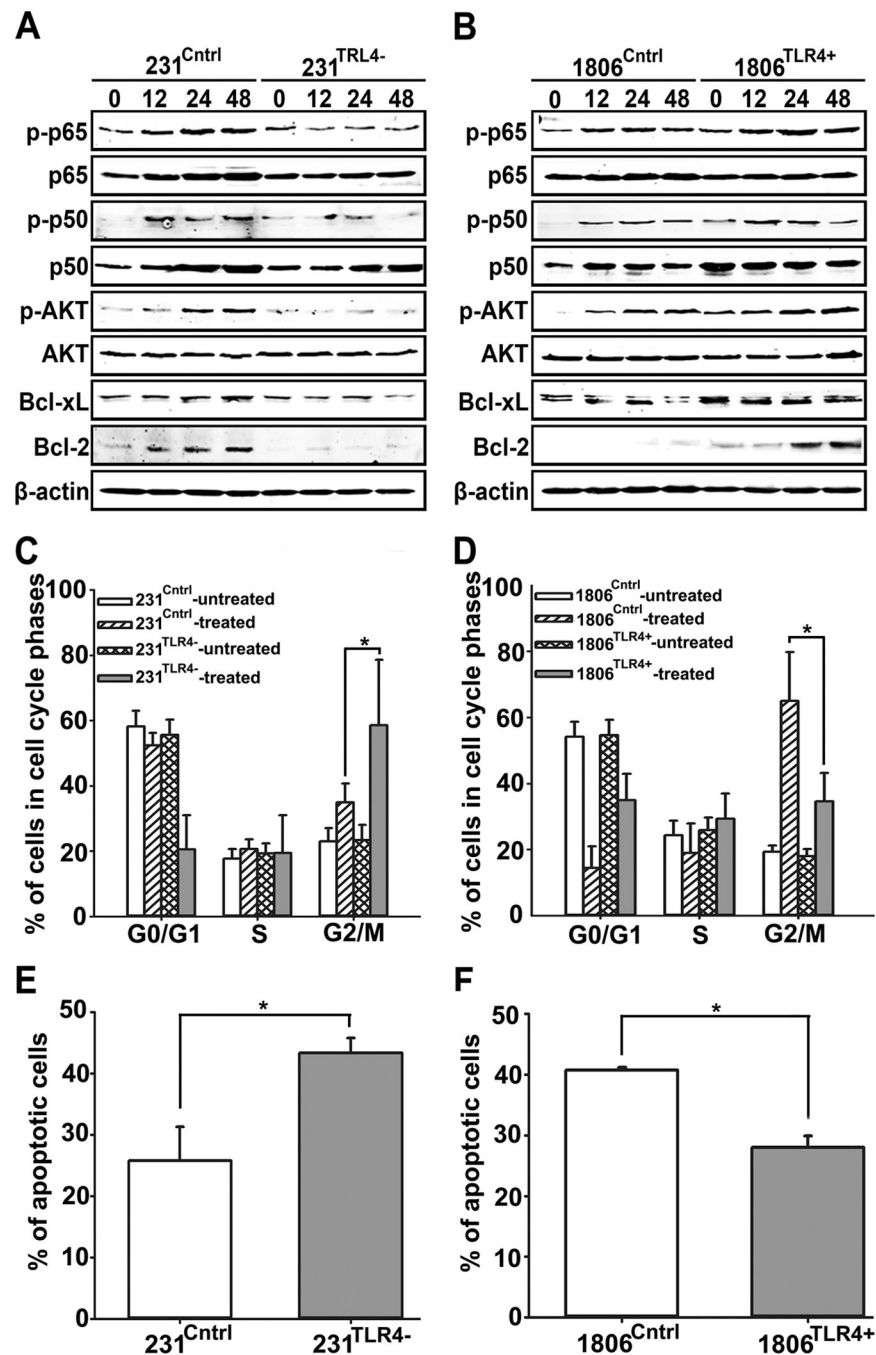
Figure 3. TLR4 mediates the response of BC cells to nab-PXL
(A, B) Western blot analysis of NF-κB p-p50, NF-κB p50, NF-κB p-p65, NF-κB p65, p-Akt, Akt, Bcl-2, Bcl-xL and β-actin proteins in 231^{Cntrl}, 231^{TLR4-}, 1806^{Cntrl}, and 1806^{TLR4+} cells treated with nab-PXL (10nM, 48hrs). **(C, D)** Cell cycle analysis by FACS of PI-stained cells treated with nab-PXL for 48hrs. Data are presented as mean percentage of cells in different stages of cell cycle ±S.D. from three experiments. **(E, F)** Apoptosis of 231^{Cntrl}, 231^{TLR4-}, 1806^{Cntrl}, and 1806^{TLR4+} cells treated with nab-PXL for 48hrs determined by FACS measurement of% Annexin-and PI-positive cells. Data are presented

as mean percentage of apoptotic cells ±S.D. from three experiments. Asterisks indicate P-values <0.05 vs. control as determined by Student's unpaired *t*-test.
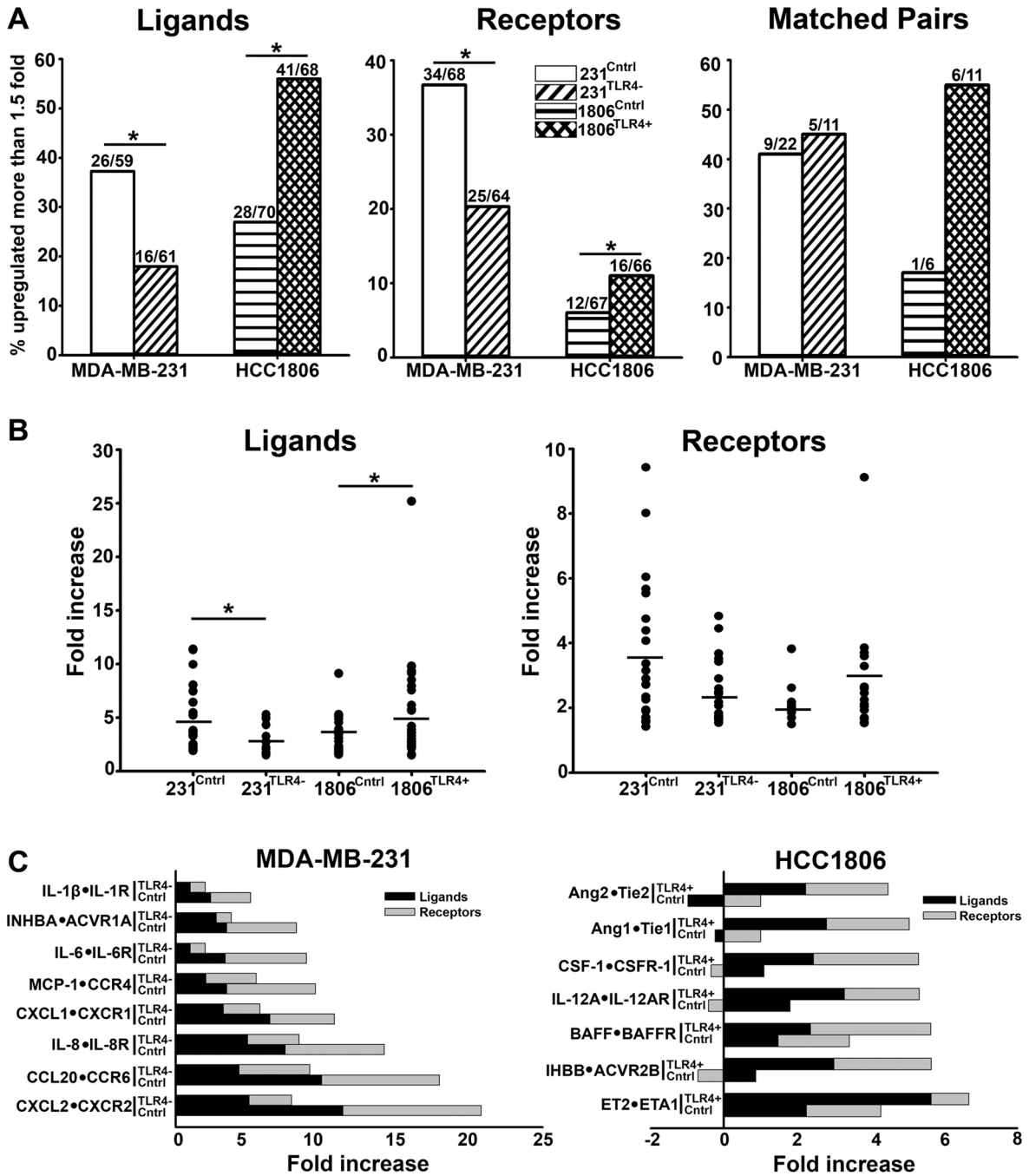
Figure 4. PXL upregulates inflammatory cytokines and receptors in TLR4+ lines
(**A**) Number of inflammatory genes upregulated >2 fold in231^{Cntr}, 231^{TLR4},1806^{Cntr} and 1806^{TLR4+} cells in PXL-treated(10nM, 48hrs) vs. untreated cells. Asterisks * and ** indicate P-values<0.05 and <0.01 vs. control determined by Chi-square test. (**B**) Fold increase of upregulated inflammatory genes in 231^{Cntrl}, 231^{TLR4-},1806^{Cntrl}, and 1806^{TLR4+} lines after PXL treatment. Asterisks * indicate P-values <0.05 vs. control as determined by Mann-U-Whitney test comparing number of ligands and receptors upregulated >1.5-fold in 231^{Cntrl} vs. 231^{TLR4-} cells. (**C**) Main cytokines and corresponding receptors upregulated >2.0 fold in 231^{Cntrl}, 231^{TLR4}, 1806^{Cntrl} and 1806^{TLR4+} cells.

A

B

C

Drug Name	Drug Type	Concentration (μM)	% Inhibition	Combination Index
Nab-PXL	Microtubule Stabilizer	0.01	45	---
C-021 dihydrochloride	CCR4 Receptor Inhibitor	1.00	13	---
C-021 dihydrochloride + Nab-PXL	-------	0.01 + 1	70	0.59
Nab-PXL	Microtubule Stabilizer	0.01	45	---
SB225002	CXCR2 Receptro Inhibitor	0.20	1	---
SB225002 + Nab-PXL	-------	0.01 + 0.20	67	0.52
Nab-PXL	Microtubule Stabilizer	0.01	45	---
PD98059	MEK Inhibitor	100	16	---
PD98059+ Nab-PXL	-------	0.01 + 100	69	0.45

D

E

Figure 5. Inhibition of CXCR2 and CCR4 receptors synergistically increases sensitivity to nab-PXL in MDA-MB-231 cells

Cells were pretreated with 0–1μM of a CXCR2 inhibitor SB225002 (**A**) or a CCR4 receptor inhibitor C021 dihydrochloride(**B**) followed by 48hrs treatment with nab-PXL(10nM). IC$_{50}$ was calculated as described in legend for Fig. 2.(C) Table demonstrating the synergy between nab-PXL and inhibitors of CCR4, CXCR2,or MEK as represented by measured as CI. (**D**) MDA-MB-231 cells were treated MEK inhibitor (0–100 uM) and nab-PXL (10nM) for 48hrs followed by calculating IC$_{50}$. Data are presented as percentage of viable cells vs. control ±S.D. from two experiments done in triplicate. (**E**) MDA-MB-231 cells were pretreated with CXCR2 (1μM) and MEK inhibitors (50μM) alone or in combination for 2hrs

followed by nab-PXL (10nM) for 48hrs. Phosphorylation of ERK1/2 and AKT was analyzed by Western blot.
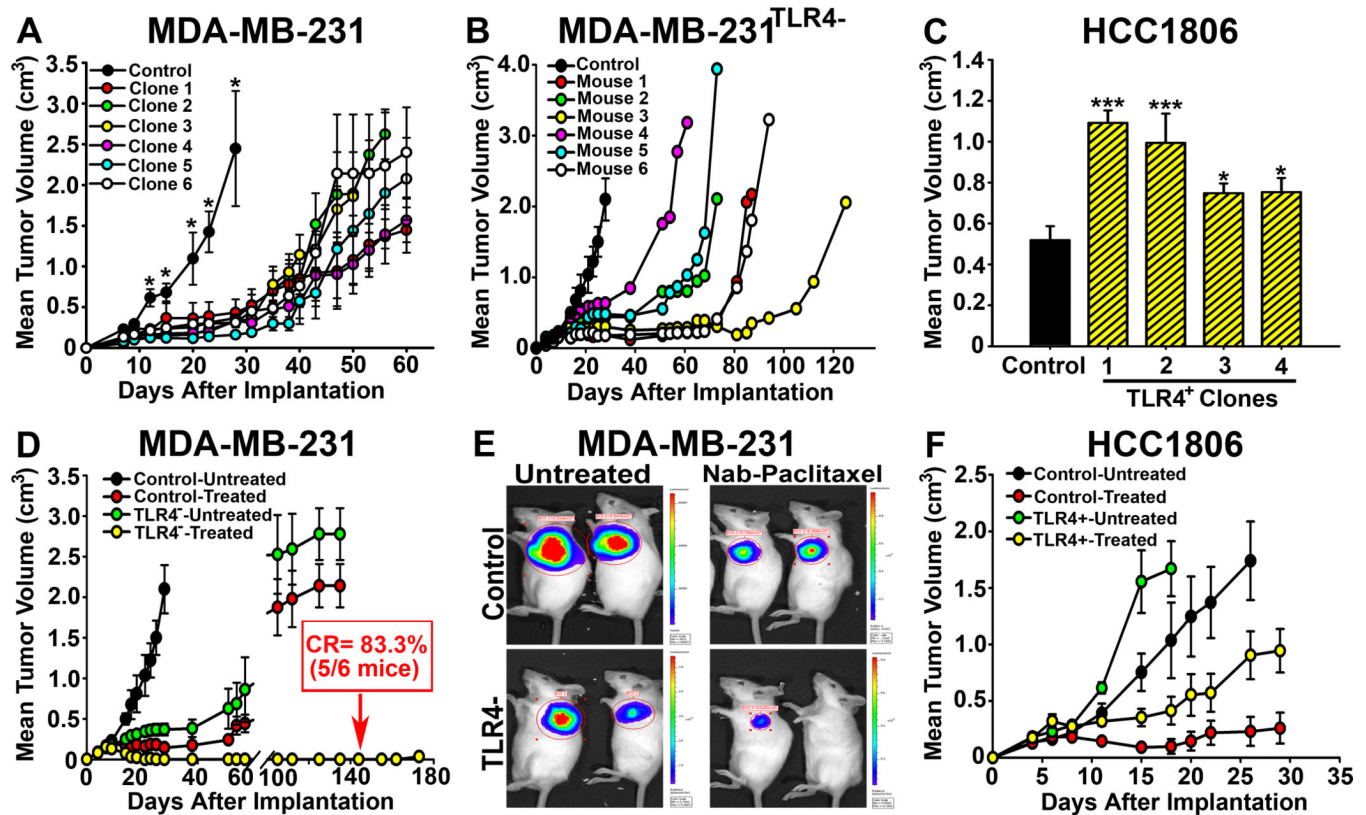
Figure 6. TLR4 determines sensitivity to nab-PXL in breast cancer models *in vivo*
(A) The growth of 231^{TLR4-} and control lines implanted in SCID mice was monitored twice weekly. Each point represents the mean tumor volume ±S.E and the asterisks indicate P-values >0.05. **(B)** Growth of a231^{TLR4-} clone demonstrating significant delay in establishing tumor mass in all mice per group (n=6) compared with controls. Each line represents tumor growth in individual mouse. **(C)** Growth of HCC1806^{TLR4+} and HCC1806^{Cntrl} tumors in SCID mice. TLR4 significantly increased tumor growth in all clones with * and *** indicating P-values <0.05 and <0.001, respectively. **(D)** 231^{TLR4-} and control tumors of 150 mm^3 were treated with 10mg/kg of nab-PXL i.v. for 8 days. Five out 6 mice (83.3%) bearing 231^{TLR4-} tumors (yellow circles) had complete response (CR) while 0% CRs was achieved in all other groups. **(E)** Bio-imaging of representative tumors from groups described in **D.** **(F)** Tumor growth of control and TLR4+overexpressing HCC1806 lines.